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Spore Heat Resistance and Specific Mineralization

GARY R. BENDER AND ROBERT E. MARQUIS*

Department of Microbiology, University of Rochester, Rochester, New York 14642

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Spores of *Bacillus megaterium* ATCC 19213, *Bacillus subtilis niger* and *Bacillus stearothermophilus* ATCC 7953 were converted to fully demineralized, but viable, H forms by controlled acid titration. H forms were more heat sensitive than were native forms, but z values were greater for killing of H spores than those for native spores. Therefore, the differences in heat sensitivity between native and H forms decreased with increasing killing temperature. The increase in heat sensitivity associated with demineralization did not appear to be due to damage to cortex lytic enzymes of the germination system because it could not be moderated by decoating heated H spores and plating them on medium with added lysozyme. H spores could be remineralized by means of back titration with appropriate base solutions. The remineralized spores, except for the Na form, were then more heat resistant than were H spores. Ca and Mn were more effective in restoring resistance than were Mg and K. Generally, the remineralized forms (except for the Na form) had z values greater than those of the native forms but still less than those of the H forms. At lower killing temperatures, the reinstatement of resistance could be related to the extent of remineralization. However, at higher killing temperatures, only a fraction of the mineral was effective in restoring resistance, and higher levels of remineralization did not result in greater resistance. Mineralization is clearly an important factor in spore heat resistance, but the relationship between resistance and mineralization is complex and dependent on killing temperature.

The resistance of bacterial spores to heat has been a focus of study for microbiologists for more than a century. The subject has tremendous practical importance in relation to sterilization and disinfection, but it also presents interesting problems in basic cell biology and biophysics. Over the past decade or so, a multi component view of the bases for spore heat resistance has developed. There appear to be three main components underlying resistance. The first is an inherent molecular component related to the evolved temperature optima for the spore-forming organisms. Thermophiles, in general, produce the most heat-resistant spores, while psychrophiles produce the least resistant spores (20). The second component of resistance appears to arise from dehydration of the spore, or more exactly, from the asymmetric distribution of water in the cell. The recent work of Beaman et al., Koshikawa et al., and Nakashio and Gerhardt (7, 12, 15) has shown clearly by use of molecular probes of permeability that most of the water within a spore is associated with integument structures, especially the cortex, while the core or protoplast is relatively dehydrated. The extent of protoplast dehydration has been correlated with heat resistance, although there appeared to be more than a single correlation modulus when a variety of spores was considered (15). Warth (21) has estimated that the apparent degree of dehydration of the spore protoplast is sufficient to account for the heat resistance of the cells.

The third component of spore heat resistance is that associated with mineralization. We have recently (13) presented data on comparative heat resistances of native and fully demineralized spores. For that work, we used procedures designed to permit full demineralization through acid extraction without detectable loss of viability in the spore populations. The data defined clearly the quantitatively important contribution of mineralization to stabilization of spores against heat killing. In this paper, we compare the heat resistances of various salt forms of spores prepared by first demineralizing the cells and then remineralizing them with specific salts. We are then able to assess the relative efficacies of specific minerals in stabilizing the spores against heat killing.

* Corresponding author.

MATERIALS AND METHODS

Spore production. Spores of *Bacillus megaterium* ATCC 19213 were harvested from shaken cultures grown at 30°C in the medium of Slepecky and Foster (19). Spores of *B. subtilis niger* were harvested from shaken cultures grown at 30°C in the complex medium described previously (13). *B. stearothermophilus* ATCC 7953 was grown at 62°C in shaken cultures in the liquid medium described previously (13), and spores were harvested shortly after the completion of sporogenesis to avoid the germination which occurs if the spores remain in the spent medium for long periods. All spores were freed of vegetative debris by repeated washing and differential centrifugation. The purified spores were stored as pellets at 4°C.

Demineralization and remineralization. Spores were demineralized by the acid titration procedures described in detail previously (13). Spores of *B. megaterium* in dense suspension in water (ca. 2.0 g [dry weight]/50 ml) were titrated with HCl over a period of about 3 h at room temperature to a pH value of 2.0 and heated briefly at 60°C to obtain complete demineralization. Spores of *B. subtilis* in dense aqueous suspension were titrated with HCl until the pH had stabilized at a value of 4.0 after about 3 h. Heating was not required to obtain complete demineralization. Spores of *B. stearothermophilus* were demineralized in a two-step procedure. First, the spores in dense aqueous suspension were titrated with HCl to a pH value of 2.0. Then, they were back titrated with NaOH to a pH value of 8.0. The resulting Na spores were then washed with water and converted to fully demineralized forms by titration with HCl to a pH value of 2.0. These procedures were designed to allow for complete demineralization without detectable loss in viability indicated by colony counts of samples plated on complex agar growth medium (see Table 1).

The demineralized H forms could then be converted to the

TABLE 1. Mineral contents of salt forms of spores

Organism	Salt form	Mineral content (μmol/mg of dry wt)				
		Ca	Mn	Mg	K	Na
B. megaterium ATCC 19213	Native[a]	0.45	0.16	0.15	0.10	0.15
	H	0.02	0.01	0.01	0.00	0.08
	Ca	0.63	0.02	0.01	0.00	0.09
	Mn	0.02	2.88	0.01	0.01	0.08
	Mg	0.02	0.02	1.27	0.01	0.07
	K	0.03	0.01	0.01	0.94	0.08
	Na	0.02	0.01	0.01	0.00	2.82
B. subtilis niger	Native[b]	0.42	0.99	0.30	0.28	0.18
	H	0.02	0.03	0.03	0.02	0.05
	Ca	0.70	0.03	0.05	0.01	0.03
	Mn	0.01	2.41	0.01	0.01	0.08
	Mg	0.01	0.03	1.41	0.00	0.06
	K	0.02	0.03	0.04	0.91	0.05
	Na	0.01	0.02	0.02	0.02	1.19
B. stearothermophilus ATCC 7953	Native[c]	0.74	0.08	0.11	0.02	0.05
	H	0.07	0.00	0.00	0.00	0.04
	Ca	1.87	0.01	0.00	0.00	0.03
	Mn	0.05	1.85	0.01	0.00	0.05
	Mg	0.05	0.01	1.04	0.00	0.03
	K	0.07	0.01	0.01	0.93	0.03
	Na	0.07	0.00	0.01	0.01	0.98

[a] Viable counting of the initial spore suspension gave an average value of 1.4×10^{10} CFU/ml. After ion exchange, spores were suspended in the original volume of fluid, and viable counts ($\times 10^{10}$) per ml were 1.5 for H spores, 1.2 for Ca spores, 3.1 for Mn spores, 5.2 for Mg spores, 1.4 for K spores, and 1.9 for Na spores.

[b] Viable counting of the initial spore suspension gave an average value of 1.5×10^{10} CFU/ml. After ion exchange, spores were suspended in the original volume of fluid, and viable counts ($\times 10^{10}$) per ml were 2.5 for H spores, 2.6 for Ca spores, 1.6 for Mn spores, 0.9 for Mg spores, 2.0 for K spores, and 1.2 for Na spores.

[c] Viable counting of the initial spore suspension gave an average value of 2.0×10^{10} CFU/ml. After ion exchange, spores were suspended in the original volume of fluid, and viable counts ($\times 10^{10}$) per ml were 2.8 for H spores, 3.8 for Ca spores, 0.7 for Mn spores, 3.9 for Mg spores, 4.0 for K spores and 3.7 for Na spores.

various desired salt forms by back titration to a pH value of 8.0, with appropriate base solutions and heating at 60°C to speed the exchange (8). Again, these manipulations could be carried out without reduction in the viability of the spore populations. The time during the demineralization and remineralization process at which the spores seem most liable to loss of viability is during any heating of H forms at 60°C, because the cells are heat sensitive in the demineralized form. The problem was mainly with *B. megaterium* because *B. subtilis* did not require heating for full demineralization and *B. stearothermophilus* is inherently more heat resistant, even in the demineralized form.

Spore minerals were extracted for assay by a procedure involving initial autoclaving of the spores in closed containers for 30 min at 126°C. HCl was then added to the autoclaved suspensions to yield a final concentration of 6 N. The suspensions were centrifuged, and the mineral contents of the supernatant fluids were assayed by atomic absorption spectrophotometry with a Perkin-Elmer model 3030 instrument. The data presented in Table 1 indicate that each of the salt forms had only a single predominant mineral and that the other minerals assayed were present in only small amounts, if at all.

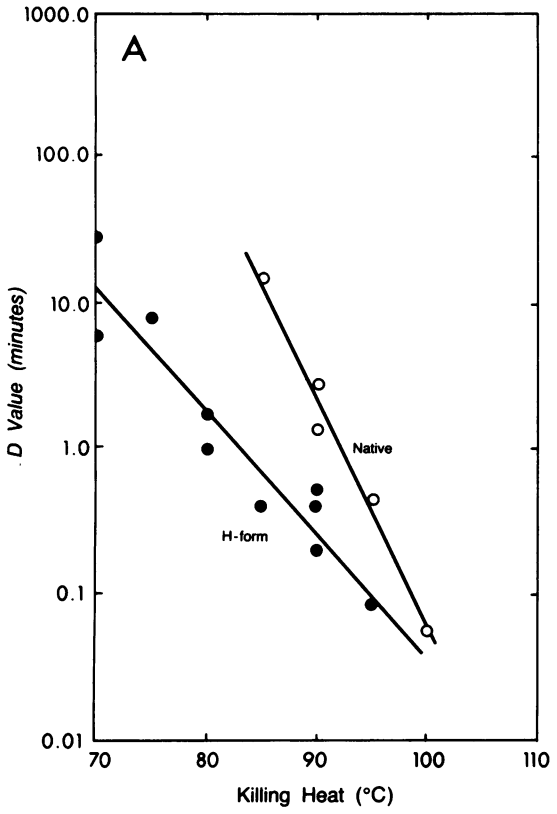
Heat sensitivity testing. For sensitivity testing at temperatures below 100°C, samples of dense suspensions with approximately 10^9 spores per ml were added directly to heated water in tubes in a temperature-controlled heating block to bring about a 10-fold dilution of the population. For testing at higher tempratures, spores in dense aqueous suspensions were sealed in capillary tubes, and the tubes were fully immersed in hot glycerol in a heating block. Samples were then cooled rapidly by transferring them directly to 1% Difco peptone broth at room temperature to bring about a 1:100 solution. The suspensions were then diluted further with 1% peptone broth, and samples were plated on tryptone-glucose-Marmite medium with 1.8% agar. The plates were incubated at the sporulation temperature for each organism. *D* values, or the times required at temperature for a one-log reduction in the spore population, were estimated from the linear portions of plots of log surviving number versus time of heating.

RESULTS

Heat sensitivities of various salt forms of spores. Figure 1 presents *D* values obtained with various salt forms of spores of *B. megaterium* ATCC 19213, *B. subtilis niger*, and *B. stearothermophilus* ATCC 7953. *D* values for native and demineralized H forms are presented for comparison. The ordinate scales in the right-hand figures have been expanded to accentuate the differences among the salt forms. The data presented in Table 1 show the mineral contents of the spores used to obtain the *D* values. The viable count figures presented in the footnotes to the table indicate that essentially full viability, within the limits of plate-counting errors, was retained in each of the test populations, despite the extensive manipulations required for full demineralization and remineralization.

As expected, *B. stearothermophilus* spores were most heat resistant, and *B. megaterium* spores were the least, with *B. subtilis* intermediate in resistance. Thus, for example, the temperature for a *D* value of 1.0 min was found to be 123.4°C for *B. stearothermophilus*, 100.1°C for *B. subtilis*, and 92.1°C for *B. megaterium*. As we and others (2, 5, 12, 16) have reported previously, conversion of spores to the H form results in marked reduction in heat resistance, and as the data of Fig. 1 show, this reduction is greater at lower killing temperatures than at higher ones. In essence, mineralization appears to be more important for heat resistance at lower killing temperatures than at higher ones. For the sproes used here, the increase in heat sensitivity associated with conversion to the H form did not appear to be due to sensitization to heat of cortex lytic enzymes required for germination as Ando and Tsuzuki (5) have found for spores of *Clostridium perfringens* NCTC 8238. If H spores were decoated by the procedure of Aronson and Horn (6) after heating and plated on medium containing lysozyme (10 μg/ml), there was no increase in plate count compared with samples plated directly on standard medium.

Remineralization resulted in increased heat resistance, except for the Na forms, and the resistances of the Ca forms were approximately equal to those of the native forms (Fig. 1). For comparison, temperatures for *D* values of 1 and 10 min are listed in Table 2. For *B. megaterium* ATCC 19213 spores, there appears to be a hierarchy of resistance with native ≅ Ca > Mn > Mg > K > Na ≅ H. Although remineralization with Ca returned the spores to a state of thermoresistance about equal to that of the native form, there still was an alteration in the temperature characteristic (μ) for killing, which decreased from 398 kJ/mol for the native form to 260 kJ/mol for the Ca form. This difference was reflected in an increase in z value from 6.3 to 9.7°C. The change in μ and z values was more pronounced for the H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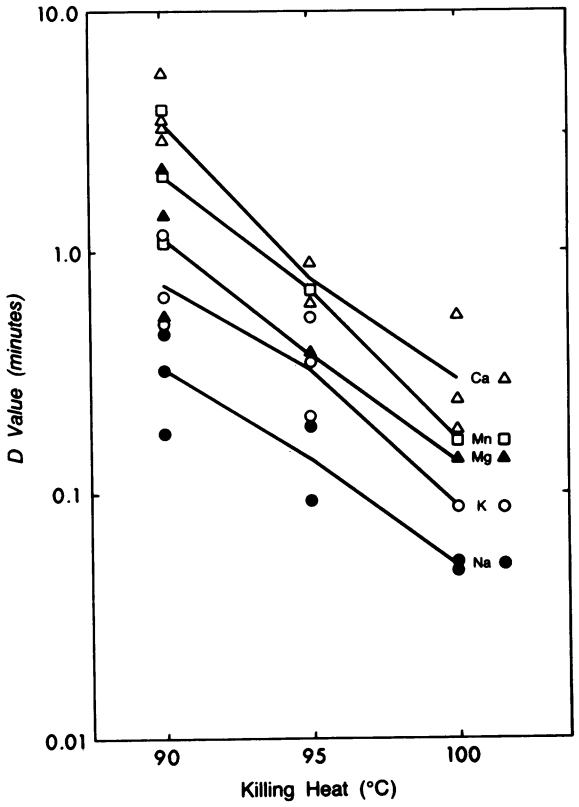
H-form
70
80
90
100
110
Killing Heat (°C)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D Value (minutes)
Ca
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Na
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95
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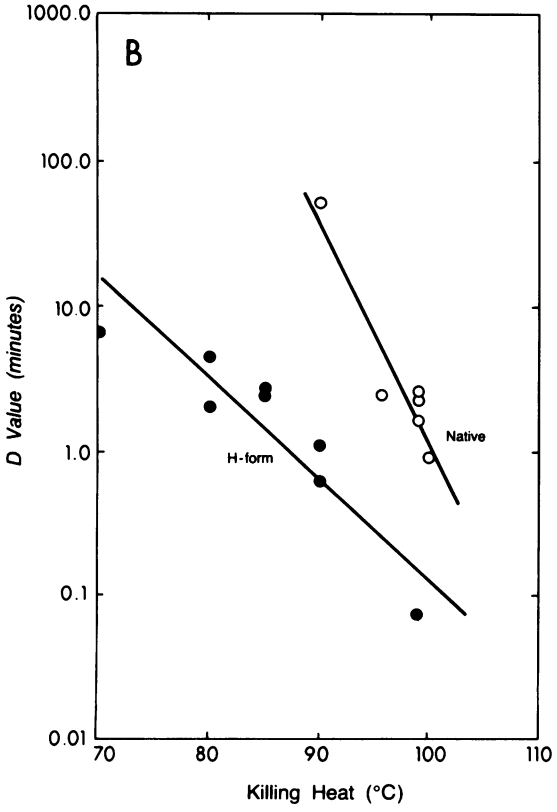
Killing Heat (°C)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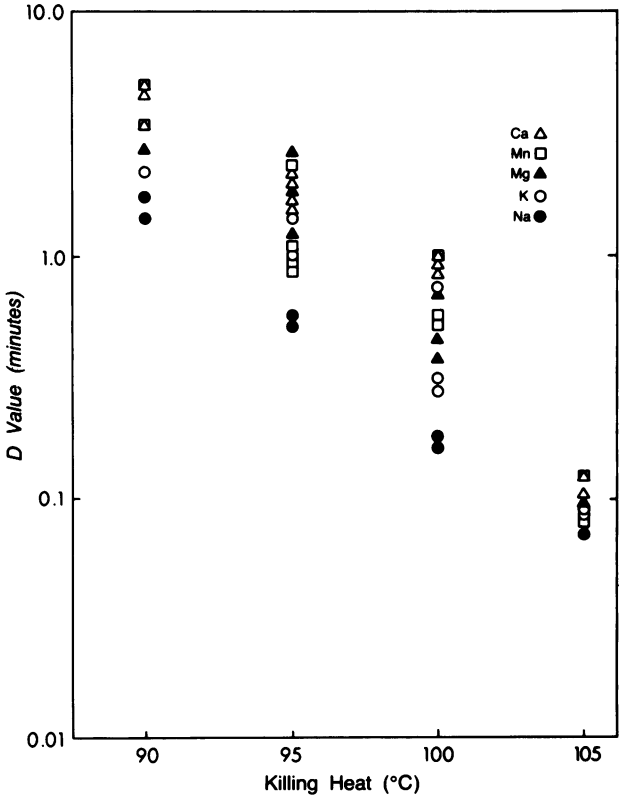
Killing Heat (°C)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D Value (minutes)
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100
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Killing Heat (°C)

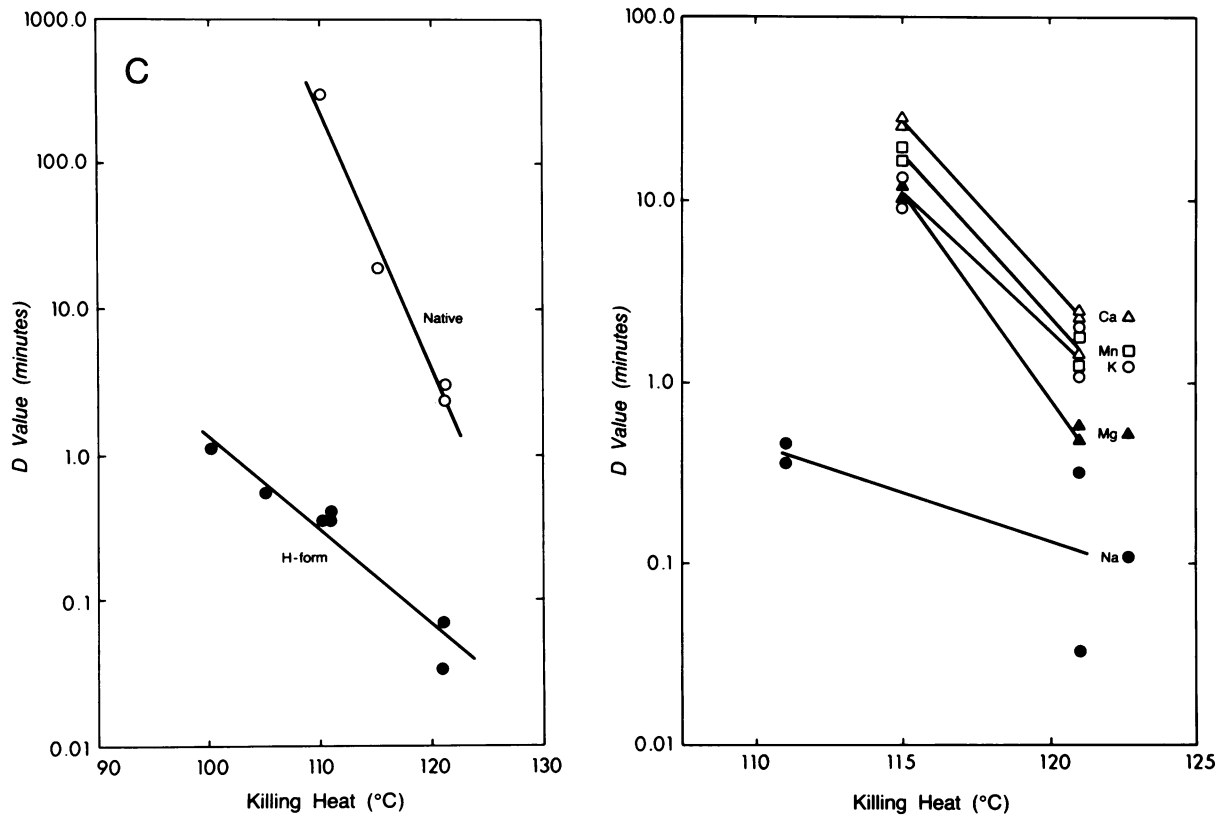


FIG. 1. *D* values for H-form, native, and remineralized spores of *B. megaterium* (A), *B. subtilis* (B), and *B. stearothermophilus* (C) plotted against killing heat.

form than for the native spores. In other words, demineralization not only reduced thermoresistance but also changed the enthalpy of activation for the killing reaction. Remineralization, especially with Ca or Mn, restored much of the thermoresistance and resulted in decreased z values (increased μ values), but the spores were not fully restored to the native state. The Na form of the spores remained as heat sensitive as the H form, and moreover, had a high z value of 15.3°C and a low μ value of 150 kJ/mol. Basically, Na spores were similar to H spores.

For calculation of μ values (equal to the enthalpy of activation $[\Delta H^{\ddagger}]$ plus RT), the following relationship was used, $\mu = [(\log D_1 - \log D_2)/(1/T_2 - 1/T_1)]\ 2.3\ R$, where D is the time required at temperature for a one-log reduction in the spore population (equal to $2.30/k$), k is the exponential death rate constant, T is the Kelvin temperature, and R is the gas constant (8.31 J/mol/K). D values of 1 and 10 min were used in the actual calculations. Technically, the plots for spore killing, such as those of Fig. 1, should be plots of log k versus $1/T$, in keeping with reaction-rate theory. However, in preparing the figures, we followed the common convention of plotting log D versus temperature.

There appeared to be a hierarchy of resistance among the various salt forms of *B. subtilis niger*, but the differences were not as large as they were with spores of *B. megaterium*, and lines were not drawn for *B. subtilis niger* in Fig. 1 because of the closeness of the points. Also, with *B. subtilis niger*, the z values in the temperature range from 100 to 105°C appeared to be less than those in the range from 90 to 100°C. Therefore, in Table 2, two estimates of z and μ are given. Again, Ca and Mn were most effective in restoring heat resistance, while Mg and K were less effective. Demineralization of *B. subtilis* spores again resulted in a marked reduction in μ value and an increase in z value, and remineralization did not entirely reverse this change.

A similar hierarchy was seen with remineralized spores of *B. stearothermophilus* ATCC 7953. Again, Ca was most effective in restoring heat resistance, and recalcified spores were essentially as thermoresistant as native spores. Na was least effective, and Na spores were essentially as thermosensitive as H spores. Mn was almost as effective as Ca in restoring heat resistance, while Mg and K were less effective. Moreover, demineralization resulted in a decrease in μ value and an increase in z value. For *B. stearothermophilus* spores, remineralization with Ca fully reversed these changes.

Thermoresistance related to extent of remineralization. The extents of remineralization of the spores used to obtain the data presented above varied, as shown in Table 1. For all three species, the Mn form of the spores was more highly mineralized than the native form, but this hypermineralization was probably due to precipitation of manganese salts at the spore surface (17). Many of the other salt forms were more highly mineralized than the native forms, possibly also as a result of salt precipitation. To assess the dependence of resistance on the extent of remineralization, we prepared mixed Ca-Na forms of the spores. The native spores were first demineralized and then remineralized in solutions with known amounts of Ca^{2+} but with sufficient NaOH to allow for titration to a pH value of 8. The spores with various amounts of Ca were then tested for thermotolerance. Results are presented in Fig. 2 in terms of D values in minutes

TABLE 2. Killing parameters for spores

Species	Salt form	Temp (°C) at which D is equal to		μ[a] (kJ/mol)	z (°C)
		1 min	10 min		
B. megaterium ATCC 19213	Native	92.0	85.7	398	6.3
	H	83.0	71.3	200	11.7
	Ca	95.0	85.3	260	9.7
	Mn	93.0	83.7	269	9.3
	Mg	90.5	79.7	227	10.8
	K	88.5	77.5	220	11.0
	Na	81.3	66.0	150	15.3
B. subtilis niger	Native	100.3	93.7	399	6.6
	H	87.2	71.8	154	15.4
	Ca	99.2	85.2	182 (490)[b]	14.0 (5.5)
	Mn	97.5	84.3	227 (415)	13.2 (6.5)
	Mg	96.5	84.0	202 (394)	12.5 (6.8)
	K	95.0	81.0	178 (382)	14.0 (7.0)
	Na	92.2	81.4	243	10.8
B. stearothermophilus ATCC 7953	Native	123.4	117.7	520	5.7
	H	102.0	86.6	167	15.4
	Ca	123.1	117.5	528	5.6
	Mn	121.9	116.7	566	5.2
	Mg	119.7	115.3	662	4.4
	K	121.8	115.5	465	6.3
	Na	104.1	85.6	140	18.5

[a] $\mu = \Delta H^{\ddagger} + RT$, where $\Delta H^{\ddagger}$ is the enthalpy of activation, R is the gas constant, and T is the Kelvin temperature.

[b] The values in parentheses were estimated from data for D values of 0.1 and 1 min.

plotted against Ca content in micromoles per milligram of spore dry weight.

For *B. megaterium* ATCC 19213 spores tested at 90°C, there was a progressive increase in D value with increasing mineralization with an indication of a plateau in resistance at higher levels of calcification. At a higher killing temperature of 95°C, the effects of remineralizaion were less dramatic, as expected, and the maximal D value was approximately the same at lower or higher levels of calcification.

The data for the inherently more thermoresistant spores of *B. subtilis niger* show progressive increase in resistance to 90°C with increasing calcification. (Note that the ordinate scale has been contracted somewhat because of the greater thermotolerance of *B. subtilis* spores.) There appears to be less tendency for D value to reach a maximum at the higher levels of calcification. At a higher killing temperature of 95°C, the effects of mineralization are again less dramatic, and there is a clear plateau in D value at calcium levels greater than about 0.45 μmol/mg of spore dry weight.

Higher temperatures were required for killing of spores of *B. stearothermophilus* ATCC 7953, but the data show a similar pattern to that obtained with the other two bacteria. At the lower killing temperature of 115°C, there was progressive increase in the D value with increase in calcification, with only an indication of a plateau in D value at high levels of recalcification. At the higher killing temperature of 121°C, the maximum D value was achieved at a Ca level of only 0.4 μmol/mg of spore dry weight, and further increases in calcification did not increase the D value.

DISCUSSION

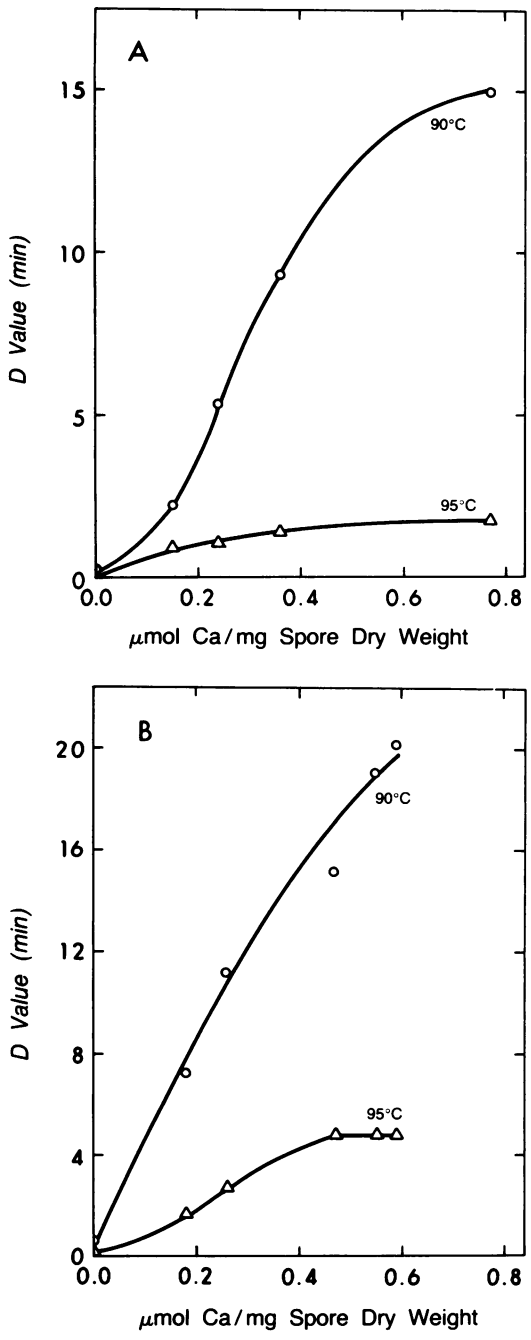
Procedures for ion exchange of spores were first developed by Alderton and Snell (2). Heat sensitivities of exchanged spores have been assessed by Alderton and colleagues (1, 3, 4), by Rode and Foster (16), by Ando and Tsuzuki (5), and by others. In general, H forms or acid-stripped spores have been found to be markedly more sensitive to heat than are native forms, and the Ca forms prepared from H forms by remineralization reacquired much of the resistance of the native forms. Ando and Tsuzuki (5) found also that acid extraction of spores of *Clostridium perfringens* NCTC 8238 caused damage to the germination system and that the increased sensitivity of the H form to heat appeared to be due to damage specifically to enzymes required for cortex lysis. Furthermore, it appeared that these lytic enzymes could be protected from heat damage by mineral ions, including sodium ions. Our findings indicate that this sort of damage did not occur in the spores we used.

Generally, in previous studies of ion exchange, detailed assays of the mineral contents of the exchanged spores were not carried out, so there is difficulty in assessing the extent of exchange. In some cases, when the pH value of the exchange medium was low, there probably was extensive demineralization. In fact, in the extreme procedures, spores were exposed to acid at pH values as low as 1.1. With the types of spores we used, this procedure would have resulted also in death of part of the population. However, in general, viabilities of exchanged populations were not monitored closely in previous studies. In our work, we routinely discarded any batches of exchanged spores for which there was a significant drop in viability to avoid problems in the interpretation of killing curves for populations with substantial numbers of dead spores initially present. In essence, the major advances reported in this paper are in methodology rather than in concept. However, the improved methodology has allowed us to come to more definite conclusions regarding the quantitative roles of minerals in heat resistance. Our main conclusion is the same as that made originally by Aldertonand Snell (2), namely, that heat resistance of bacterial spores can be affected greatly by changes in mineralization. Thus, resistance is not entirely an inherent property of the spore but can be manipulated chemically. Moreover, our analyses show that ion-exchange processes can involve all of the minerals within spores and not just those in integumental structures.

The details of the stabilization of spores to heat damage by minerals are puzzling and not interpretable in terms of known stabilization of isolated biopolymers by salts. There does appear to be a hierarchy for stabilization. Generally, Ca is most effective, but other minerals especially Mn, also are effective. The exception was Na. It was surprising that Ca was not more effective relative to the other minerals. Clearly, in assessing the relationship between mineralization and heat resistance, one must consider the total set of minerals in the spore and not just the Ca content.

For example, *B. megaterium* ATCC 19213 can be compared with *B. subtilis niger*. We found previously (13) that vegetative cells of the two bacteria had about the same heat sensitivities, with D values of 1 min at 52 and 50°C, respectively. In contrast, the H spores of *B. subtilis* were more heat resistant than those of *B. megaterium*, with D values of 1 min at 87 and 83°C, respectively. Native, mineralized spores of *B. subtilis* were more heat resistant by a still wider margin than were those of *B. megaterium* with D values of 1 min at 100 and 92°C, respectively. Presumably, this latter difference is due to the greater mineralization of native spores of *B. subtilis* and mainly to their higher Mn, Mg, and K contents since both types of spores had about the same Ca content.

B. stearothermophilus spores had a higher Ca content than



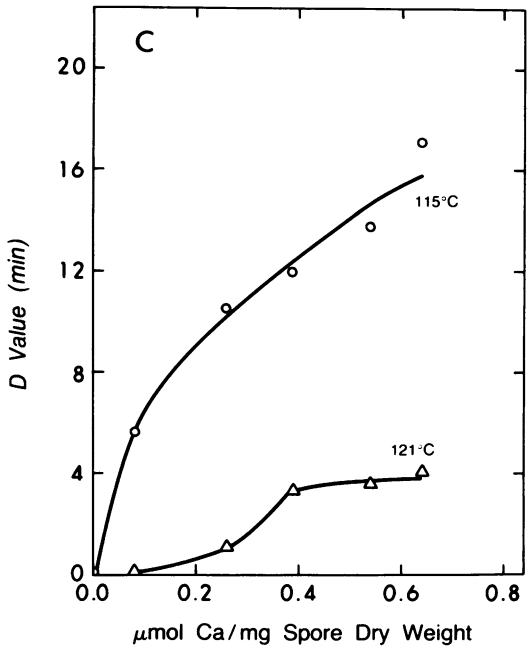


FIG. 2. *D* values for spores of *B. megaterium* (A), *B. subtilis* (B), and *B. stearothermophilus* (C) demineralized and then remineralized with limiting amounts of calcium.

did spores of the other two test bacteria. Moreover, vegetative cells and mineral-free H spores of *B. stearothermophilus* were more heat resistant. Thus, all three main components of resistance may be involved in the greater resistance of *B. stearothermophilus* spores. There is greater inherent or molecular resistance related to the higher temprature range for growth of the organism. There is a component of resistance related to greater calcification, although *B. subtilis* spores actually had greater total mineralization because of their higher contents of Mn and Mg. Finally, it appears from the data for H spores that another part of the greater resistance of *B. stearothermophilus* has to do with the spore state, probably with the greater dehydration of the protoplast of the spore of *B. stearothermophilus* reported by Nakashio and Gerhardt (15).

The effects of mineralization on z values and on the enthalpies of activation ($\Delta H^{\ddagger}$) for spore killing were surprising, although previously, Alderton et al. (1) did find differences in z values for native, H-form, and recalcified spores of *Clostridium botulinum* 62A. For example, at a water activity of 0.9, z values were 7.4°C for native spores, 9.4 for acid-stripped spores, and 13.6 for acid-stripped, recalcified spores. Unfortunately, spore killing is complex and probably involves multiple processes. The decrease in enthalpy of activation for killing associated with demineralization could be the result of a change in the biochemical basis for killing,

example, as Ando and Tsuzuki (5) found for acid-stripped spores of *C. perfringens* NCTC 8238. However, we have not been able to detect damage to the cortex-lytic enzymes of the spores we used, and the most reasonable interpretation for the change in enthalpy involves a multi step killing process.

Heat killing of spores is usually considered mainly in terms of irreversible heat denaturation of proteins, although the exact basis for killing of any particular spore is far from clear at this time, and the recent work of Hanlin and Slepecky (10) suggests that heat damage to DNA may be important for killing. The current view of protein structure features dynamic molecules with multiple conformations in aqueous environments. For enzymes, only some of these conformations are catalytically active. Denaturation is viewed as a multistep process with more than one molecular conformational route to the irreversibly denatured state. Protonation of proteins could limit the numbers of paths to denaturation or change the overall enthalpy of activation of certain routes. Thus, protonation could render the proteins more susceptible to heat denaturation but limit the process to conformational routes with lower enthalpies of activation. For many proteins, acidification lowers the enthalpy of activation for denaturation (11). Generally, this decrease is accompanied also by a decrease in the entropy of activation so that the net free energy of activation is not changed greatly because of acidification. Thus, the decrease in the enthalpy of activation for killing of H spores may be interpreted in terms of protein denaturation. However, it is then difficult to interpret the low enthalpy of activation for the killing of Na spores along the same lines.

For some proteins, the changes induced by protonation may not be entirely reversible, at least not in the time span of our experiments, and so remineralization would not result in a complete change in the value for the enthalpy of activation back to that characteristic of the native state. Moreover, a mix of minerals may be required to reconstitute fully the native state. The changes appear not to involve loss of dipicolinate because complete demineralization occurred without appreciable loss of dipicolinate, assayed by the method of Scott and Ellar (18). Much of the dipicolinate could be extracted subsequently by heating the demineralized spores at 50 or 60°C, but it is clear that minerals and dipicolinate are extracted separately from the spores we used. It seems then that ion exchange is truly exchange and not extraction. Thus, during acidification protons move into the spores to displace Ca and other minerals. The dipicolinate must then become protonated, or possibly, complexed with positively charged groups in proteins or other biopolymers. At the pH value of the exchange reactions, most of the protein carboxyl groups would become protonated, and so the proteins would have a net positive charge due to positively charged amino groups. Presumably, the spore membranes must become sufficiently permeable during exchange to allow mineral ions to pass from the core to the cortex and from the cortex across the coats to the exterior.

The exact mechanisms by which minerals stablilize spores against heat killing are not known. The mechanisms must not be entirely dependent on salt bridging by divalent cations because K^+ is effective as a stabilizer, although less so than the divalent cations tested. In addition, we found previously (14) that demineralization and remineralization do not change appreciably the overall state of hydration of the spores as indicated by the dextran-impermeable volumes of the cells per unit of dry weight. Ionic size may be a pertinent factor for stabilization by minerals. The ionic radii of the divalent cations Ca^{2+}, Mn^{2+}, and Mg^{2+} are 0.099, 0.080, and 0.065 nm, respectively. The ionic radii of K^+ and Na^+ are 0.133 and 0.095 nm, respectively. Thus, both charge and size may be important for stabilization. The ionic radii given here are those of the dehydrated ions. Stabilization potential could not be related to the radii of the hydrated ions. The apparent hierarchy for stabilization does not follow the Hofmeister series or any of the other series described for the interactions of mineral ions with soluble proteins. There is a possibility that the stabilization by minerals is mainly of biopolymers not in aqueous solution. Indeed, minerals are effective in stabilizing dried spores against heat damage (1), and so water is not required for stabilization, although it may affect the process. The extremely low mobilities of core ions reflected in the dielectric properties of spores (9) suggest that many of the heat-labile biopolymers of the cells may be precipitated with mineral ions. In fact, stabilization may require precipitation.

Perhaps our most surprising finding is that mineralization is more important for spore heat resistance at lower killing temperatures than at higher ones. This difference is reflected in the slopes of the lines in Fig. 1 for H spores and native spores and also in the curves for *D* value versus Ca content of Fig. 2. For example, for *B. subtilis*, the *D* value at 90°C could be increased by some 20 min through remineralization of the H spore, while at 95°C, the *D* value could be increased by only about 5 min. Moreover, at the higher temperature, the maximum stabilizing effect was achieved at a lower level of remineralization. Again, it seems that the results are best interpreted in terms of multiple molecular routes to inactivation, with at least one route with a low value for $\Delta H^{\ddagger}$ which is not much affected by demineralization.

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